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Nutritional Effect of Some Cholestenols and Cholestadienols on the Silkworm *Bombyx mori*

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Several cholestenols and cholestadienols were tested for ability to support the growth of the silkworm, *Bombyx mori*. 5α-Cholest-6-en-3β-ol completely fulfilled the insect sterol requirement, whereas 5α-cholest-8(9)-en-3β-ol and (20*Z*)-5,20(22)-cholestadien-3β-ol were unable to sustain larval growth at all. The other sterols examined were partially effective as nutrients.

Keywords——insect sterol; membrane sterol; silkworm; *Bombyx mori*; 5α-cholest-6-en-3β-ol; 5α-cholest-9(11)-en-3β-ol

The silkworm, *Bombyx mori*, is an excellent laboratory tool for investigation of the biochemical role of sterols, because the larvae can be reared on artificial diet supplemented with an appropriate sterol. Supplementation with cholesterol (**1**), sitosterol[(24*R*)-24-ethylcholesterol] or campesterol[(24*R*)-24-methylcholesterol] allowed good growth and development of the larvae.[1] The latter two sterols are major sterols in mulberry leaves, the natural diet of *B. mori*, and they are dealkylatively metabolized in the insect to cholesterol,[2] a principal and probably functional sterol of the silkworm. Various intermediates of this dealkylation also satisfied the sterol requirement.[3] In order to investigate the sterol structure-biological function relationship, several cholesterol analogs were also examined as possible

TABLE I. Effect of Cholestenols and Cholestadienols on the Growth and Development of the Silkworm *Bombyx mori*

Compound	Number of larvae in the indicated instar			Average weight (mg)
	1st	2nd	3rd	
5-Cholesten-3β-ol (**1**)	0	1	39	46
5α-Cholest-6-en-3β-ol (**3**)	0	9	31	37
5α-Cholest-8(9)-en-3β-ol (**20**)	3	3	0	4
5α-Cholest-8(14)-en-3β-ol (**8**)	0	38	2	24
5α-Cholest-9(11)-en-3β-ol (**21**)	3	5	0	4
(20*E*)-5α-Cholest-20(22)-en-3β-ol (**9**)	0	22	18	23
5α-Cholesta-7,9(11)-dien-3β-ol (**10**)	0	34	0	15
5α-Cholesta-7,14-dien-3β-ol (**11**)	0	40	0	19
5α-Cholesta-8,14-dien-3β-ol (**22**)	0	0	0	0
(20*E*)-5,20(22)-Cholestadien-3β-ol (**12**)	5	33	0	18
(20*Z*)-5,20(22)-Cholestadien-3β-ol (**19**)	5	0	0	3
(22*E*)-5,22-Cholestadien-3β-ol (**13**)	0	35	4	32
(22*Z*)-5,22-Cholestadien-3β-ol (**14**)	2	33	0	12
5,23-Cholestadien-3β-ol (**15**)	0	39	0	22
5,25-Cholestadien-3β-ol (**16**)	0	26	12	22

The number and average weight of surviving larvae on day 15, starting with 40 newly hatched larvae, are shown.

insect nutrients. It was found[4] that cholesterol analogs containing a side chain with 5 to 10 carbon atoms could sustain growth, *albeit* less effectively than cholesterol. Further, the silkworm can tolerate certain modifications of the sterol nucleus, since it was demonstrated[5] that 5α-cholest-7-en-3β-ol (**2**), 5,7-cholestadien-3β-ol (**5**) and 6,8(14)-cholestadien-3β-ol (**6**) can replace cholesterol as sterol sources for *B. mori*, and fairly good growth was also obtained with 5α-cholestan-3β-ol and 5α-cholest-14-en-3β-ol (**7**). To obtain further insight into the effect of particular double bond(s) in the cholestane skeleton on silkworm growth, we have now tested various cholestenols and cholestadienols as possible insect nutrients.

According to the previous method,[3-5] the test sterols were added to an artificial diet, on which newly hatched silkworm larvae were reared. The growth and development are recorded and the results are summarized in Table I. The only sterol containing a double bond in the cholestane skeleton which allowed as good growth as was observed with cholesterol, was 5α-

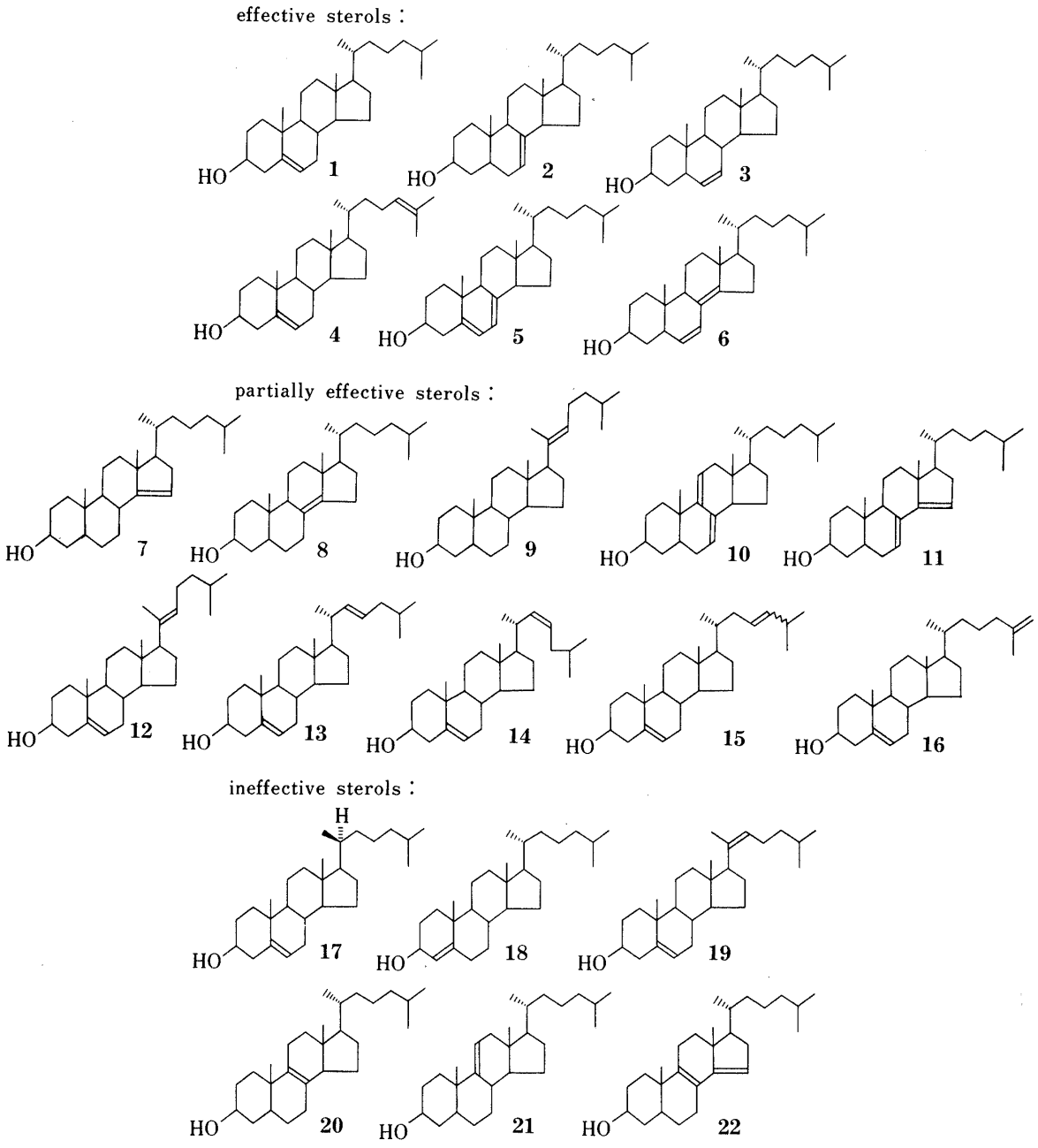

Fig. 1. Classification of Cholestenols and Cholestadienols Based on Their Nutritional Effect in *Bombyx mori*

cholest-6-en-3β-ol (**3**). The other sterols tested were either partially effective or were unable to support silkworm growth at all. Figure 1 shows a classification of cholestenols and cholestadienols examined in the present and previous[5] experiments, in terms of ability to act as a silkworm nutrient. The fact that Δ^6- and Δ^7-cholestenol (**3** and **2**) are "effective" sterol seems to suggest that the double bonds at C-6 and C-7 can functionally substitute for the Δ^5 double bond of cholesterol. In contrast, Δ^4, $\Delta^{8(9)}$ and $\Delta^{9(11)}$ double bonds were highly deleterious to larval growth. $\Delta^{8(14)}$-, Δ^{14}- and $\Delta^{20(22)}$-Cholestenols (**8**, **7** and **9**) behaved similarly to 5α-cholestan-3β-ol,[5] and therefore these double bonds are neither beneficial nor injurious.

The characteristics of the conjugated cholestadienols appear to be predictable from the results with the isolated double bond compounds just described. For example, the $\Delta^{5,7}$-diene (**5**) may be regarded as a combination of Δ^5 and Δ^7, and so this sterol is expected to be an "effective" sterol. Combination of an effective Δ^6 with a "partially effective" $\Delta^{8(14)}$ in the $\Delta^{6,8(14)}$-diene (**6**), similarly permitted good growth *albeit* at a slightly reduced rate compared to the $\Delta^{5,7}$-diene (**5**). The partial effectiveness of the $\Delta^{7,9(11)}$-cholestadienol (**10**) and $\Delta^{7,14}$-cholestadienol (**11**) may result from a combination of effective Δ^7 with "ineffective" $\Delta^{9(11)}$ or partially effective Δ^{14}. $\Delta^{8,14}$-Cholestadienol (**22**), which combines the deleterious effect of $\Delta^{8(9)}$ with the partial effectiveness of Δ^{14}, behaves as an ineffective sterol.

The influence of an additional double bond in the side chain of the cholesterol skeleton on larval growth was next examined. Double bonds at C-20, -22, -23 and -25 induced more or less retardation of silkworm growth. In contrast, desmosterol (**14**) was previously shown[3] to permit good growth, but this may be due to its metabolism to cholesterol by C-24, 25 hydrogenation.[2] Comparing the *E* and *Z* isomers of 20- and 22-dehydrocholesterol, the latter

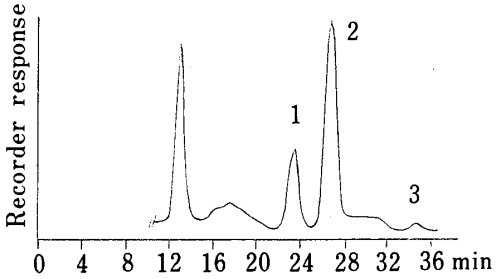

Fig. 2. Gas Chromatogram of Insect Sterol Trimethylsilyl (TMS) Ethers

The larvae of *B. mori* (40 species) were reared on 5,25-cholestadien-3β-ol for 15 d. The unsaponifiable fraction of lipid extract was treated with TMS imidazole (20 μl) and the TMS ethers were analyzed with a Shimadzu-LKB 9000S gas chromatograph-mass spectrometer using 1.5% OV-17 (1 m) at 245 °C. The retention times and mass spectra of peaks 1, 2 and 3 coincided with those of the authentic TMS ethers of cholesterol, Δ^{25}-Cholesterol and sitosterol, respectively.

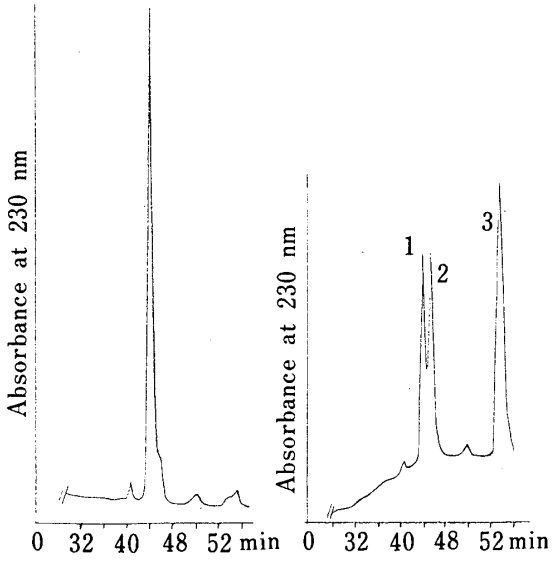

Fig. 3. High Pressure Liquid Chromatography of Insect Sterol Benzoates [Left] and of Authentic Benzoates of Δ^6-Cholestenol (Peak 1), Cholesterol (Peak 2) and Sitosterol (Peak 3) [Right]

The sterol TMS ether of *B. mori* fed 5α-cholest-6-en-3β-ol was prepared and analyzed by GC-MS as mentioned in the legend to Fig. 1, and the remaining sample was hydrolyzed in a mixture of CH_2Cl_2 (1 ml) and methanol (1 ml) containing conc.HCl (2 μl) for 0.5 h at 20 °C. The resulting free sterol was converted to the benzoate as described previously[8] and analyzed with a Shimadzu LC-4A high pressure liquid chromatograph using Zorbax ODS (25 cm × 4.6 mm i.d.) with methanol at a flow rate of 1.5 ml/min.

isomer appeared to be more deleterious in both cases. (20*Z*)-20-Dehydrocholesterol (**19**) was completely ineffective, and no larvae fed this sterol molted to the second instar. This result for the 20*Z*-olefin (**19**) is similar to that observed with 20-isocholesterol (**17**),[6] and therefore the stereochemistry around the C-20 position of sterol appears to be of crucial importance for satisfying the insect sterol requirement.

It is interesting to note that some of the larvae fed 20*E*-, 22*E*- or 25-dehydrocholesterol developed to the second or even to the third instar, even though hydroxylation at C-20, -22 or -25, which is an essential process for ecdysone biosynthesis, is blocked in these compounds. We may consider three possibilities to account for this. 1) The molting hormone utilized in these insects might be structurally modified from the conventional ecdysone. 2) Ecdysone could conceivably be biosynthesized from the olefinic test sterols *via* hydrogenation and hydroxylation. 3) The artificial diet contained not only the test sterol (0.1%) but also phytosterol (0.003%, a mixture of sitosterol and campesterol) present in soybean oil, which is one of the components of the diet; thus, the ecdysone in these insects might come not from the test sterol but from the phytosterol. Further work is required to decide among these possibilities.

To determine the effect of sterol structure on silkworm growth, it is of critical importance to know whether dietary test sterols were metabolically transformed into other sterol(s), *e.g.* cholesterol. For this purpose, we analyzed insect sterols as their trimethylsilyl (TMS) ethers by gas chromatography-mass spectrometry (GC-MS) as described previously.[7] The major sterols were always the test sterols, and other sterols always amounted to less than 5% except in the case of larvae fed 25-dehydrocholesterol (**16**). The latter larvae contained, as shown in Figure 2, *ca.* 20% cholesterol apparently produced from **16** by hydrogenation. It should be noted that Δ^6-cholestenol (**3**) TMS ether was not separated from cholestenol TMS ether on GC, and further, their mass spectra were indistinguishable from each other. Therefore, the sterol of the larvae fed **3** was analyzed by high pressure liquid chromatography in the form of the benzoate ester.[8] It can be seen from Fig. 3 that the principal insect sterol was **3**, together with *ca.* 3% cholesterol. The origin of this cholesterol (either Δ^6-cholesterol or phytosterol) remains to be clarified.

In summary, none of the test sterols except for Δ^{25}-cholesterol (**16**) was extensively metabolized into other sterol(s). If we assume that phytosterol in the diet could serve as an ecdysone precursor, the test sterol incorporated into the insects might be utilized (or not utilized) solely as a cell membrane constituent. It follows that the nutritional effects of the test sterols mentioned above may be reflecting the structural adequacy of the cholestenols and cholestadienols for membrane functions.

Experimental

The sources of sterols used in the present experiments were described in our previous paper.[8] Insect rearing was carried out as described previously.[3-5] Analysis of insect sterols was performed on day 15 after hatching essentially in the same manner as described previously.[7] For details, see the legend to Fig. 2.

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